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The overall aim of this project is to improve our understanding of genetic factors regulating the development, differentiation, function, and neoplastic progression of the breast. In 1997 we have investigated the first clearly identified mammary phenotype in homeobox genes, an engineered mutation in mouse *Hoxd-10* that causes a deficiency in milk production. We report that *Hoxd-10* is strongly expressed in the mammary epithelium in a stage-dependent manner, with highest levels found in late pregnancy and lactation. Analysis of the phenotype is in progress, but at present it appears that the defect lies in functional differentiation. Failure to differentiate is a hallmark of neoplastic progression. A second area of reported research is investigation of the hedgehog signaling pathway, a potential upstream regulator of *Hoxd-10*, other homeobox genes, and a variety of mammary-active genes. We report data showing strong expression of members of the mammalian hedgehog pathway, several of which are known oncogenes. Experiments are in progress using engineered mutations, as well as investigations of the biological activity of the hedgehog protein itself in the mammary gland.

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C. W. Jamil Jan 29, 1998

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

The highlights of our 1997 research year were (a) the discovery of the first mammary phenotype associated with a homeobox gene and (b) identification in the mammary gland of a highly expressed upstream regulatory pathway with the potential to regulate these and other mammary-active genes. In collaboration with Prof. Ellen Carpenter, UCLA School of Medicine, we have identified a mammary defect caused by targeted disruption of the mouse *Hoxd-10* gene. As will be discussed, there are indications of roles for other homeobox genes, but a demonstrated requirement for *Hoxd-10* is in itself a major advance, justifying research funding by the DOD and pointing toward future directions for homeotic gene research in mammary gland biology.

The general aim of this project has been to obtain a better and ultimately more clinically useful understanding of the genetic mechanisms underlying development of the normal breast and of the initiation, progression, and spread of breast cancer. The reasoning behind this question is as follows. The breast is a target organ for a variety of hormones. These, together with growth/differentiation factors, regulate the activities of the mammary cell. Unfortunately, this does not take us very far in understanding the biology of this interesting organ. Consider simply that other organs are also regulated by these same signaling molecules, but develop by quite a different pattern. The mammary gland itself varies enormously between species, between individuals, and of course in malignancy. How can this variation be accounted for, when the signals are the same? There must exist additional layers of genetic regulation that interpret these signals and give rise to particular patterns of development, or to neoplasia. How do we search for these developmental regulatory genes? In organisms such as *Drosophila*, where detailed genetic analysis is possible, mutations provide clues that have led geneticists to identify gene families that act as master regulators of cell fate, determining for example, whether a wing or an antenna will develop at a particular location. The discovery of these regulators has had an enormous impact on thinking in biology.

One such group of genes is the homeotic family, which act as transcription factors, switching on or off groups of genes that specify the details of developmental processes. One of the triumphs of molecular biology during the past decade has been the discovery that these "homeobox" genes are not limited to the fly, but are ubiquitously distributed and remarkably conserved. In the mammals, including the human, not only do they occur in the genome, but their numbers and types are greatly amplified. It is now well established by gene targeting in the mouse that these genes are essential for determining many aspects of early embryogenesis, and more recently, it seems that they may be active in the development of tissues and organs, and that their malfunctions may contribute to cancer.

Do homeobox genes influence the breast, and if so, could they contribute to cancer? This is the question underlying our research, and during the first year we discovered that many of these homeobox genes are active during the growth and development of both the mouse mammary gland and human breast. Their patterns of expression are frequently altered in cancer, some overexpressed, others underexpressed or not expressed at all. Because these genes serves such central regulatory roles, these discoveries create optimism that new insights into mammary development will be a product of these studies, and that an entirely new class of mammary oncogenes or tumor suppresser genes may be shown to exist.

During the second year of research we studied the human breast, using surgical samples, and found similar patterns of expression. In the course of these investigations we

discovered a new multi-gene family in the human breast, designated *IRX* in recognition of the Iroquois Related gene family discovered at about the same time in Drosophila.

Our approach is to use the mouse mammary tumor model system for those observations and experiments that describe the patterns of expression of these genes. In addition, the mouse model, with its capability of "reverse genetics," provides the potential to explore the role of homeotic genes in mammary development and in the etiology of cancer. These experiments cannot be done in humans, of course, but we have simultaneously carried out expression studies on normal and malignant breast samples and compared them with findings obtained with the mouse. In addition, we are cloning human homeobox genes that cannot be obtained elsewhere, and it was in connections with this that the previously mentioned IRX family was discovered.

In 1997 our research was highlighted by a shift in emphasis from expression studies of multiple genes to more narrowly focused investigations of the functional roles of selected genes. This change was self-initiated, but it was also suggested as a result of the reviews of our 1996 annual progress report. Listed below is the Revised Statement of Work, submitted and accepted in Fall 1997:

REVISED STATEMENT OF WORK (Changes in italics)

Task 1. Homeobox gene expression in mouse mammary gland, Months 1-48.

a. Using Northern and *in situ* molecular hybridization, and immunohistochemistry as appropriate, we will explore expression of homeobox genes for the duration of the grant. Expression patterns discovered in this continuing series of experiments will feed into other tasks.

b. Special attention will be given to the IRX family of homeobox genes, recently discovered in our studies on homeobox gene expression in the human breast

c. Special attention will be given to genes shown in Task 4 to have a functional role in the mammary gland.

Task 2. Homeobox expression in human breast cancer. Months 1-48.

a. Months 1-12. Obtain tissue samples from approximately 10 patients, prepare mRNA stocks.

b. Months 1-12. Study expression of *Hoxa-1* and *Hoxa-2*

c. Months 13-48. Continue expression studies in genes indicated in mouse studies in Task 1.

Task 3. Homeobox regulation. Months 1-48.

a. Months 1-12. Whole animal studies of ovariectomy and replacement therapy.

b. Months 12-24. Estrogen/progesterone studies using implants.

c. Months 12-48. Growth factor studies. Transgenic experiments, implant studies.

d. *Regulation of homeobox genes by upstream regulatory pathways.*

Task 4. Functional studies. Months 1-48.

a. Months 1-*48*. Optimize cell transplantation protocols. *For Hoxa-1 and IRX-2, prepare in vitro transfected and adenoviral vector infected cells for transplantation.*

b. Months 12-*48*. Infect, select, and transplant cells. Evaluate phenotype and establish cell lines, if appropriate.

c. *Examine the null phenotype of mice in which Hoxd-9 and Hoxd-10 have been disrupted by gene targeting.*

Justification.

Both reviewers comment on substantial progress and speak favorably about the importance of the research. The first reviewer recommends that the SOW be revised to reflect the increased importance given to the IRX gene family. The second reviewer comments on a lack of focus, and recommends concentrating on a small number of genes.

Task 1b, 1c. These tasks have been added to give specificity and selectivity to this study. Task 1b reflects our discovery of robust, highly specific, developmentally regulated expression of the IRX gene family and our conviction that it will have important implications for mammary development and cancer. Task 1c indicates that expression studies will be carried out of those genes shown in Task 4 to have a functional role, using gene targeting and transfection.

Task 3d. This task has been revised to delete the retinoic acid studies and substitute investigations of upstream regulatory genes. Our early experiments have shown this to be a more productive approach. Specifically, we have excellent evidence for the existence of a hedgehog signaling pathway in the mammary gland, in which Hedgehog peptides (*Hh*) signal through the Patched receptor (Ptc) to activate the family of Gli transcription factors, which in turn influence the expression of several homeobox genes, including members of the Hoxd cluster, as well as other key mammary regulators.

Task 4. Minor additions to Task 4a reflect technical changes in our gene transfer procedures.

Task 4d has been added to describe an additional approach to functional studies of homeobox genes, that could not have been envisioned when the original proposal was prepared. Through a series of collaborations we have obtained engineered mice in which Hox d10 has been disrupted through gene targeting. The d10 mutants appear to display a mammary phenotype. There is additional evidence that *Hoxd9/Hoxb9/Hoxa9* triple heterozygotes have a similar phenotype. We have initiated an active breeding program to produce both null and heterozygous mice for study. We will confirm and describe the phenotype, and carry out further studies as described in Tasks 1-3.

BODY OF NARRATIVE

Objective 1. *Homeobox gene expression in mouse mammary gland, with emphasis on the IRX family and on genes which have been shown to have functional activity in the mammary gland.*

The IRX Family

Our 1996 progress report contained a detailed description of this work. Reviewers of the manuscript suggested that additional data confirming the existence of multiple family members be supplied. Accordingly, we obtained additional sequence data and also carried out southern analysis, showing multiple forms containing the IRX homeobox. Additionally, other laboratories have confirmed these findings. Cloning and expression studies in embryonic tissues of the IRX family have been carried out in the laboratory of C.C. Hui (Toronto), who has generously shared probes and sequence information with us.

Similar data has been reported from the laboratory of P. Gruss (Germany). All of these laboratories have had difficulty in obtaining complete sequence data from IRX genes, due to unusually high G-C content, but nevertheless the data appear to be consistent and point towards active future investigation in this area.

Hoxd-10: expression and functional analysis.

Because expression data (Task 1) and functional studies (Task 4) are tightly linked in the case of Hoxd-10, these are considered together.

Phenotype. In a recent report, Carpenter et al (1997) reported that targeted disruption of Hoxd-10 produces mice with hindlimb-specific defects in gait and adduction. To determine the underlying causes of this locomotor defect, mutant mice were examined for skeletal, muscular and neural abnormalities. Mutant mice exhibit alterations in the vertebral column and in the bones of the hindlimb. No major alterations in hindlimb musculature were observed, but defects in the nervous system were evident. There was a decrease in the number of spinal segments projecting nerve fibers through the sacral plexus to innervate the musculature of the hindlimb. Deletion of a hindlimb nerve was seen in some animals, and a shift was evident in the position of the lumbar lateral motor column. These observations suggest a role for the Hoxd-10 gene in establishing regional identity within the spinal cord.

As an indirect consequence of our collaboration with Dr. Capecchi (Salt Lake City) we were contacted by Dr. Carpenter, who described an apparent mammary defect in these mutants and proposed a collaboration. We obtained breeding pairs of Hoxd-10 heterozygotes and began an extensive breeding program. In addition, we have developed a small colony of athymic mice to be used as hosts in transplantation studies of these and other engineered mice. A germ-free surgical facility for these immunologically challenged mice has also been established in our animal facility and is now in routine use .

Our initial phenotypic analysis of female mice homozygous for a disrupted *HoxD10* gene identified a defect in lactation. Pups from early litters of mutant females died from a lack of milk, but survived if pups were fostered with lactating wild type females. Lactational failure appears to be most pronounced in the first litter, and becomes less severe in subsequent litters, such that multiparous breeders are able to nurse successfully. At least three hypotheses could explain this defect: 1) glands are developmentally delayed, 2) glands are defective in lobule-alveolar differentiation, and 3) glands are defective in functional differentiation (lactogenesis) such that milk production and secretion is compromised.

We have initiated a program to characterize this phenotype and obtain insights into the functional activity of Hoxd-10. A breeding program has been set up to permit examination of mice at all stages of mammary development and during the lactation cycle. In addition, we have transplanted gland from mutant females (-/-) into nude hosts, such that one inguinal cleared fat pad is transplanted with mutant tissue and the contralateral fat pad with control gland from wt littermates. This will permit comparison of growth, differentiation, and lactation against a uniform hormonal and physiological environment. This powerful approach will also permit us to investigate the role of Hoxd-10 in mammary epithelium vs the peri-glandular stroma, since the glands resulting from these transplants are chimeric, in which the stromal component is always wt.

To date we are only beginning to obtain the mice needed for analysis. Morphological studies of glands from virgin mutant females and from early stages of pregnancy do not indicate a development retardation, indicating that the defect probably lies

in cell differentiation or functional activity. We have not yet been able to examine these stages, will be able to do so in the coming weeks. Similarly, the transplant experiments are well underway but animals for analysis will be not be available for another month. A defect or delay in differentiation can have implication for the cancer problem, since this phenotype would maintain cells in the proliferating pool for a longer period with consequent increased opportunity for neoplastic changes.

Temporal patterns of expression. The discovery of a *Hoxd-10* mammary phenotype indicated an urgent need for detailed expression data. These studies are still underway but already we have obtained interesting data indicating robust expression levels of the gene in the mammary gland. Significantly, the highest levels are found in late pregnancy and lactation. This is an unusual pattern, since expression patterns of other Hox genes generally shows high levels during ductal development and early pregnancy, but low to undetectable amounts in lactation.

We investigated the normal expression pattern of the *Hoxd-10* gene by both Northern blot analysis and in situ hybridization. We have examined gland development in homozygous mutant and age-matched wild type control animals at many critical stages. These include: immature (5 week), mature (12-13 weeks), early pregnant (7.5 d.p.c.), late pregnant (17.5-19.5 d.p.c.), lactation (6 hours and 6 days), involution (days 2, 10 and 14), and lactation in a second pregnancy. In addition, we will soon be able to examine each of these stages (except 6 day lactation and involution) in contralateral transplants of mutant and wild type tissue in cleared fat pads of athymic (nude) mice.

Expression of Hoxd-10 examined by northern analysis

Table 1. Northern blot (polyA RNA) using a gene-specific probe to Hoxd-10. Band densities were standardized against the ribosomal protein L7.

	5 wk	mature	EP	LP	Lact*	2 day Involl	10 day Invol	14 day Invol
HoxD10	+	++	+++	++	++	ND	ND	ND

*Expression during lactation must be evaluated in light of the dilution effect resulting from large amounts of milk protein transcripts. In our experience and that of others, even moderate levels detected during milk secretion indicates very high steady-state transcript levels.

Spatial patterns of expression. We have used hybridization *in situ* with gene-specific *Hoxd-10* probes to detect expression patterns in various stages of mammary development. In 5 week animals, *Hoxd-10* expression is concentrated in body cells of terminal end buds and is slightly reduced in cap cells (Fig. 1A). Considerable expression is also observed in the periductal stroma of the subtending duct. This expression pattern is maintained in mature glands which also show reduced expression in myoepithelial cells relative to lumenal epithelial cells (Fig. 1B). During pregnancy, lobule-alveolar cells also express *Hoxd-10* (Fig. 1C and D). In late pregnancy, alveolar epithelium begins to express at higher levels than in ducts (Fig. 1D). Highest levels of *Hoxd-10* expression are observed during lactation, in which 100% of alveolar epithelial cells stain darkly early in the color development (Fig. 1E). During involution (data not shown) expression is undetectable at 2 days and returns as early as 10 days. At 14 days involution, the pattern of expression is comparable to that observed in the mature virgin. Sense strand control hybridizations show

no staining, indicating specificity of antisense probe binding. These *in situ* hybridization results are consistent with the northern hybridization results.

Summary and conclusions. The ongoing experiments reported above represent a significant advance over earlier studies, in the sense that they begin with a phenotype, rather than exploring for one. Comprehensive breeding and transplant programs are underway, and interesting results are all but assured. Our expression studies will soon be completed, and show a pattern in which expression levels increase with progress through the lactation cycle, reaching a climax during secretory activity. This expression pattern strongly supports the phenotype.

Objective 2. Homeobox expression in human breast cancer.

Our progress in this area, particularly with the *IRX* gene family, was described in the 1996 Progress Report. The revised SOW added one component to this, namely expression studies on *HOXA-2*. Again as previously described, the mouse homolog of this gene was found to be expressed at high levels in tumors, but not in normal gland or in pre-cancerous tissues from which the tumors were derived. This suggested that if the gene has a role in cancer, it may be in the later stages of mammary cancer progression, characterized by increased malignancy.

We have made strenuous efforts to obtain gene-specific probes for human *HoxA-2*, but so far without success. We will use RT-PCR if necessary, and if expression is detected, we may clone a portion of the gene for the production of probes to be used for *in situ* analysis.

Objective 3. Regulation of homeobox genes by upstream regulatory pathways.

Introduction. This objective, added in the 1997 revised SOW, is a direct outcome of two of our recent findings. First is the discovery of an apparent mammary role for Hoxd-10, and second is cloning and expression of the IRX gene family in the gland. In model systems, all of these genes are regulated, at least in part, by the hedgehog signaling pathway. An investigation of this pathway in the mammary gland was a logical and interesting extension of this previous research. Although a great deal of attention has recently been focused on hedgehog signaling in various developmental systems (reviews: Altaba, 1997; Hammerschmidt et al, 1997), it has not been studied in the mammary gland to our knowledge, and certainly nothing has been published.

An outline of hedgehog signaling in Drosophila, where it was first discovered and investigated in detail, is shown in Fig. 2A. A single hedgehog ligand (*Hh*) is implicated in both short-range and long-range signaling through its receptor patched (*ptc*), whose activity is modified by another membrane protein, *Smo*. Hedgehog signaling is mediated by cubitus interruptus (*Ci),* a putative transcription factor that regulates downstream homeotic genes such as members of the Iroquois family (Gomez-Skarmetaet al, 1996), decapentaplegic, wingless, and patched itself. In the fly the hedgehog pathway has many essential functions, is active in many locations, and is reactivated at various times in development, from early segmentation and axial patterning to development of structures such as the wing, leg, eye, in the larva.

In vertebrates the pathway is not only conserved, but new family members have been added with a resulting increase in complexity and developmental plasticity (Fig 2A).

In mammals and birds hedgehog has been expanded to include Sonic hedgehog (*Shh*), Indian hedgehog (*Ihh*), and Desert hedgehog (*Dhh*), whereas *Ci* has been expanded to include a family of three vertebrate transcription factors, the *Gli* genes, so named because of their initial identification and cloning from a glioblastoma (Rupperet et al, 1988). In addition to *Gli*, *Ptc* has recently been linked to both inherited and sporadic skin cancers, which include the basal cell carcinoma, the most common human cancer (Johnson et al, 1996). In spite of the expansion of family members, the signal transduction cascade appears to be remarkably conserved (Fig 2A).

The multiple functions of the hedgehog pathway in vertebrate development are being studied in several laboratories and it is evident that, as in the fly, hedgehog regulation of developmental process occurs in many locations and in many developmental periods. One the most fully documented and elegant examples is in the developing limb, where Shh secretion regulates patterning of the anterior-posterior axis (Marigo et al, 1996). Numerous developmental genes have been shown to be regulated by this pathway (Fig 2A) such as IRX, Hox, TGF-Beta, BMP, FGF, and even the recently discovered parathyroid-related-protein (PTRP), which in gene targeting experiments has recently been shown to be essential for embryonic growth of the mammary gland (J. Wysolmersky, personal communication). The above list reads like a litany of genes and cell products that are known to be important to mammary development and cancer. If it is eventually found that the hedgehog pathway influences only a fraction of these in the mammary gland, that will be an important discovery.

Research Plan. Our approach to studying the hedgehog pathway in the mammary gland involves three strategies. The first is expression, using appropriate combinations of molecular probes and antibodies as available. Second, we have obtained mutants of various components of the pathway and are well into a breeding program that will enable us to examine phenotypic changes in development, function, or neoplastic potential of the gland. These studies will make use of our nude colony, enabling us to examine mutant and control tissues in a uniform physiological environment. Third, we have made arrangements with Ontogeny, Inc., Cambridge MA to supply us with several micrograms of functional *Shh* protein. This will be incorporated into slow-release plastic implants, using techniques we have pioneered (Silberstein and Daniel, 1987), which will be implanted directly into the mammary gland of test mice. These experiments are extremely well controlled in that glands on the contralateral side of experimental animals receive implants containing carrier protein only, again making possible meaningful comparisons of even subtle effects.

Materials and Methods.

(1) *Mice.*

Two breeding pairs of mice carrying a transgene consisting of *Ptc* under the control of the metallothionine promoter (mtPtc) were obtained from Dr. M. Scott. The transgene was originally carried in a FVB/CD1 F1 background. Homozygotes were generated by intercrossing of heterozygotes at various points in the breeding program.

Two breeding pairs of mice heterozygous for a disrupted *Gli2* gene were obtained from C.C. Hui (Toronto). The mutation was originally maintained in a CD1 background and maintained in our laboratory as an interbreeding population with periodic backcross to CD1.

Two breeding pairs of mice heterozygous for a disrupted *Gli3* gene ($Gli3^{XtJ}$ allele) originally maintained in a C3H/CD1 F1 background were obtained from C.C. Hui (Toronto). A colony was established in our laboratory using these pairs followed by

intercrossing of their progeny. Subsequently, heterozygous progeny were backcrossed to CD1. Homozygotes were generated by intercrossing of heterozygotes at various points in the breeding program.

The inbred mouse strain Balb/C is maintained in our laboratory and was used for all expression analyses to maximize consistency of results and allow direct comparison of data and correlation to the large number of genes whose expression is already characterized in this strain. Athymic (nude) mice were obtained from Simonsen. CD1 mice were obtained from Charles River Laboratories. C57/DBA2 F1 mice were obtained from Taconic.

For pregnancy and lactation studies, mice were matured to 10 weeks of age and mated. Stage of pregnancy was counted with the morning of appearance of a vaginal plug as 0.5 d.p.c. and confirmed by staging of embryos at the time glands were removed.

Developmental stages examined: The following developmental stages were used: 3 week, 5 week, mature (10-12 week), early pregnant (6.5-7.5 d.p.c.), late pregnant (17.5-19.5 d.p.c.), lactating (6 hours), lactating (6-15 days), early involution (2 day), mid-involution (6-10 day), late involuting (14 day). Not all stages were investigated with each technique, as noted.

Transplants into nude mice and analysis of results: Fragments (~1 mm^3) of #3 or #4 mammary gland were transplanted contralaterally into cleared #4 fat pads of athymic mice. The developmental stages used are those described for the whole gland analysis of intact animals (above) except for >6 hours lactation, which is not possible to study in transplanted tissue because there is no nipple connection to maintain secretion. Two sets of littermate mice are used and three transplants per stage per transplant set are examined. For *Gli2* and *Gli3*, +/+ and -/- tissue was harvested from E19.5 embryos and used to establish tissue lines in a primary transplant. Secondary transplants from these outgrowths were used to examine the behavior of these tissues at different developmental stages. For *Ptc*, homozygous tissue cannot be obtained since embryos die very early in development. Since intact +/+ and +/- animals can be examined directly, tissue was used in a long term study to examine whether or not the +/- tissue formed tumors more quickly than the +/+ contralateral controls. Such long term studies are not possible in intact animals since the heterozygotes generally die of brain tumors before other tumors arise. Mice swill be periodically palpated for tumors and were harvested after 1 year (October 1998).

Northern Hybridizations, RTPCR, in situ hybridizations. immunostaining, and whole gland analysis were carried out as previously described in the DOD proposal.

Slow-release Implants containing active Shh: Implants are made as described (Silberstein and Daniel, 1987)) and contained 4ug SHH-N per 1mm^3 implant. The following test tissues are used to assess effects on development: 5 week, mature, ovx, ovx+E+P, 5 week+E+P. Pellets are implanted in front of growing endbuds in 5 week animals and ovx animals; implants were placed in the central region of the gland in mature animals.

Organ culture: To assess effects in pregnancy and lactation is may be desirable to use an organ culture system. The following test tissues will be used: mature, late pregnant, and lactating. Tissues will be examined in the presence of 2uM Shh-N, 2uM Shh-N preincubated with + anti-Shh-N, anti-Shh-N alone, and no treatment. The amount of anti-Shh-N required to ablate SHH-N activity will be determined by titration using primary

mammary epithelial cells isolated from a +/- *ptc* heterozygote and chemiluminescent assay for induction of the ptc:LacZ fusion.

Results.

Data from breeding programs and transplants is only just starting to appear. Expression studies are, however, in full progress and we have obtained solid evidence of robust expression of members of the hedgehog pathway.

Temporal patterns of expression using RT-PCR

Dhh. Desert hedgehog was seen to be present in all mammary tissues examined by RT-PCR (Figure 2B). When the round one RT-PCR reactions were electrophoresed on a 2% ethidium bromide gel, bands of the correct size, 791 nucleotides, were observed. A Southern Blot was performed and these products hybridized to a Desert hedgehog probe done under high stringency conditions (not shown).

Ihh:. Indian hedgehog was shown to be expressed in mammary gland at stages 3 week, 5 week, mature, early pregnant, late pregnant, and 4 day involuting mammary tissue and in the 14 day embryo positive control (Figure 2B). The round one RT-PCR products were of the expected size (565 bp) and hybridized to an *Ihh* probe in a Southern Blot of the RT-PCR products (not shown).

Shh: In a round one RT-PCR reaction we detected no visible PCR products on a 2% ethidium bromide gel from the sampled mammary gland RT reactions (not shown). A Southern blot of the gel did however detect PCR product of the correct size in 5 week, early pregnant, late pregnant, and lactating mammary gland (Figure 2B). The positive control 14 day embryo RT yielded a visible band of expected size (570 bp). This also corresponds with a round two nested PCR reaction performed using material from the round one PCR reactions (Figure 2B).

Temporal patterns of expression using Northern hybridizations

Ptc: Patched expression was detected on a Northern blot comprised of total RNA collected from different developmental stages of the mouse mammary gland (Fig. 2C). Two transcripts were observed at 9 kb and 8.2 Kb. There was no notable difference in the levels of expression between the two transcripts in relation to each other; both were either present or absent in each tissue sample. Expression was highest in 5week tissue and slightly lower in ovariectomized mice. Low levels of patched expression were detected in mature; 11 day and 14 day pregnant. *Ptc* expression was not detected on this total RNA Northern blot in 17 day pregnant, lactating, 4 day involuting or epithelium cleared mammary gland. A chart comprised of ratings of patched expression levels (3 given to the highest level of expression and 0 to no detection on this Northern) is shown in figure 2C.

Ptc expression was also observed in cancerous and precancerous tissues examined using a total RNA Northern blot. Fourteen day pregnant normal tissue showed a low level of expression of *Ptc* while the two different precancerous hyperplastic alveolar nodules (HANs) examined, D1 and D2 showed a comparatively high level of expression. Four tumors derived from each HAN (total of eight tumors) were examined and in all cases expression of patched was reduced with respect to the HAN from which it was derived. The patched transcripts observed were the same sizes as those seen on the developmental blot. Expression was scored on a scale from 0 (not detectable) to 4 (highest level) and plotted for each sample (Figure 2C)

Gli : Expression of *Gli* in the mammary gland was examined using the same developmental Northern blot mentioned above. Two transcripts were detected at 4.4 Kb and 4.0 Kb. There was no notable difference in the levels of expression between the two transcripts in relation to each other, and both were either present or absent in each tissue sample. Gli expression was highest in ovariectomized and 4 day involuting tissue and lower in 5 week and mature mammary glands. Low levels were detected in 11 day and 14 day pregnant and no *Gli* expression was seen in 17 day pregnant, lactating, or epithelium-free cleared tissue. Expression was scored from 0 (not detectable) to 4 (highest level) and plotted for each sample. (Figure 2C) *Gli* expression in cancer progression has not yet been examined.

Gli 2: Expression of *Gli 2* in the mouse mammary gland was examined using the same developmental Northern blot mentioned above. One transcript was detected at 8 Kb. The highest level of expression was found in 5 week, followed by ovariectomized, and low detection of *Gli 2* was seen in the mature mammary gland. No *Gli2* expression was detected in any of the pregnant stages, lactating, involuting, or cleared tissue (Fig. 2C). The expression of *Gli2* with regards to cancer progression was examined. The highest expression of *Gli2* detected was in the D2 HAN followed by slightly lower expression in the D1 HAN. Expression was observed to be significantly reduced in all eight tumors derived from these nodules. Expression was scored from 0 (not detectable) to 4 (highest level) and plotted for each sample (Fig. 2C).

Gli3 was shown to be expressed in the mouse mammary gland using the same developmental Northern blot. One transcript of 9 Kb was detected with highest expression in 5 week, followed by reduced expression in ovariectomized, mature, 11 and 14 day pregnant. Some *Gli3* expression was seen in 17 day pregnant. No *Gli3* was detected in lactating, 4 day involuting, or cleared.

In cancer progression *Gli3* expression was highest in the D2 HAN and went down in the D2 tumors derived from that same HAN outgrowth line. In contrast, expression of *Gli3* in the D1 HAN was somewhat higher than two of the D1 tumors, about the same than one of the D1 tumors, and less than in another one of the D1 tumors. Fourteen day pregnant gland showed a similar expression level to two D1 tumors and three of the D2 tumors. Expression was scored from 0 (not detectable) to 4 (highest level) and plotted for each sample (Fig. 2C).

Spatial patterns of expression using in situ hybridization and immunohistochemistry:

Ptc: By *in situ* hybridization, in terminal endbuds of 5 week animals *ptc* is expressed primarily in body epithelial cells and is noticeably reduced in cap cells (Fig. 3A). In the subtending duct, expression is maintained in lumenal epithelium but undetectable in myoepithelium. Some expression is also observed in the periductal stroma of ducts. This pattern is maintained in mature ducts (Fig. 3B). In early pregnancy, expression is elevated in developing alveoli relative to ducts (Fig. 3C). In late pregnancy, ductal and lobule-alveolar expression is maintained (Fig. 3D). Expression appears to be highest during lactation with alveolar epithelium staining very darkly early in the course of color development. (Fig. 3E) At early involution, expression drops to undetectable levels (Fig. 3F) and begins to be re-established at least as early as 6 days of involution (Fig. 3G).

Levels comparable to the mature virgin appear late in involution when the gland is largely remodeled (Fig. 3H).

Expression at the RNA level is qualitatively recapitulated at the protein level. The highest degree of immunostaining appears during lactation (Fig. 3I) and detection at all stages is blocked by preincubation of the antibody with the peptide used as the antigen (Fig. 3J). Staining is distinctly membrane associated, as expected for this receptor.

Whole gland analysis:

Ptc: Preliminary analysis of glands from *Ptc* +/- animals used for embryo harvest is completed. These animals were of various ages and were either pregnant 7.5 d.p.c. or plugged but not pregnant. One +/+ control animal was also examined which was pregnant 7.5 dpc. Ages ranged from 3-7.5 months. Similar lobule-alveolar structures were observed in both sets of animals, though the frequency and degree to which lobule-alveolar structures were developed appears to be slightly greater in the ptc +/- animals regardless of whether or not the animals were actually pregnant. This may be a function of animal age. We have also examined C57/DBA2 control animals at 5 weeks, 12 weeks, and 18 d.p.c. and observe small lobule-alveolar structures though not as highly developed as those in some *Ptc* +/- animals.

Transplants into nude mice:

Ptc: in progress. Preliminary transplants of +/- epithelium showed that transplanted tissue does not form unusual structures in virgin animals. For the long range tumor study, no tumors were detectable at 3.5 months.

Gli2: In preliminary experiments, epithelium from -/- newborn animals was successfully transplanted and formed glands in adult athymic hosts with a morphology consistent with the gland structure of the virgin host animal. Crosses are in progress to establish both +/+ and -/- transplant lines form littermate animals to be used as the source material for the full developmental analysis.

Gli3: In preliminary experiments, epithelium from -/- newborn animals (a male, in this case) was successfully transplanted and formed glands with a morphology consistent with the gland structure of the virgin host animal. Crosses are in progress to establish both +/+ and -/- transplant lines from littermate animals to be used as the source material for the full developmental analysis.

Objective 4. Expression of homeobox genes in situ

Functional analysis of expressed homeogenes is being carried out by overexpressing homeogenes in mouse mammary gland and examining the resulting phenotype *in situ.* Preneoplastic and neoplastic changes, as well as alterations to normal growth patterns will be related to expression. In the case of the mouse mammary gland a unique variation on these techniques is available. Mammary epithelium cultivated as a monolayer can be transfected *in vitro,* where selection can be used to enrich the cultures for cells carrying introduced genes for antibiotic resistance. These cells are injected into a gland-free fat pad where they are capable of regenerating mammary gland. Even small variations in growth rate, pattern, or functional activity of these regenerated glands can be readily recognized by experienced observers. Areas of interest can be selected and

transplanted into other fat pads, creating tissue lines of genetic variants that can be tested for incidence of preneoplastic and neoplastic changes.

These experiments are in progress. Control vectors carrying the lac-z reporter have been used successfully, and our monolayer cultures can be transplanted into the gland-free fat pads of isogenic mice with a 80-90% success. Gland transfected with *Msx-2* and *Hoxa-1* have been incorporated into retroviral vectors, propagated in producer cells, and the harvested viruses used to infect mammary epithelial cells in culture. Our initial results were disappointing, with relatively poor efficiency in gland regeneration and no detectable phenotype. These experiments are being repeated with minor modifications designed to improve outgrowth regeneration and we shall have data in a few weeks.

Summary and Conclusions

The investigation of hedgehog signaling described above is a logic consequence of earlier experiments on *IRX* and *Hoxd* genes. Although these studies are very much in progress, data of several types indicates strong expression of pathway components. This is the first time that this pathway has been demonstrated in the mammary gland, and it has important implications for development, lactation, and carcinogenesis. Indeed, if upcoming experiments with mutants and with administration of *Shh* ligand show that the pathway is not only expressed but is functional, this has the potential to considerably influence thinking on mammary regulation, and to provide a conceptual scaffold for making certain predictions concerning the upstream regulation of a variety of downstream mammary-active factors.

Of the three mammalian forms of hedgehog, *Shh* is the most commonly expressed, particularly in the embryo, and the *Shh* mutant gives the most severe embryonic lethal phenotype. *Dhh* is associated primarily with the male genital system and *Ihh* with gut development and chondrocyte differentiation. It would be expected, therefore, that *Shh* would be the dominant form in the mammary gland. Our expression data suggests however that *Ihh* is expressed at higher much levels. This is based on PCR data, which is semi-quantitative at best, but that is the consistent finding. Northern analysis of *Gli* isoform expression shows interesting patterns of stage-specific expression, and differences between normal, preneoplastic HAN, and tumor that could be significant. It must be noted that there is apparent contradiction between the northern and *in situ* data with regard to expression levels during lactation. Northern blots are unreliable in lactation due to the great dilution effect with milk protein transcripts. The heavy signal seen with both *in situ* hybridization and immunostaining is therefore more reliable.

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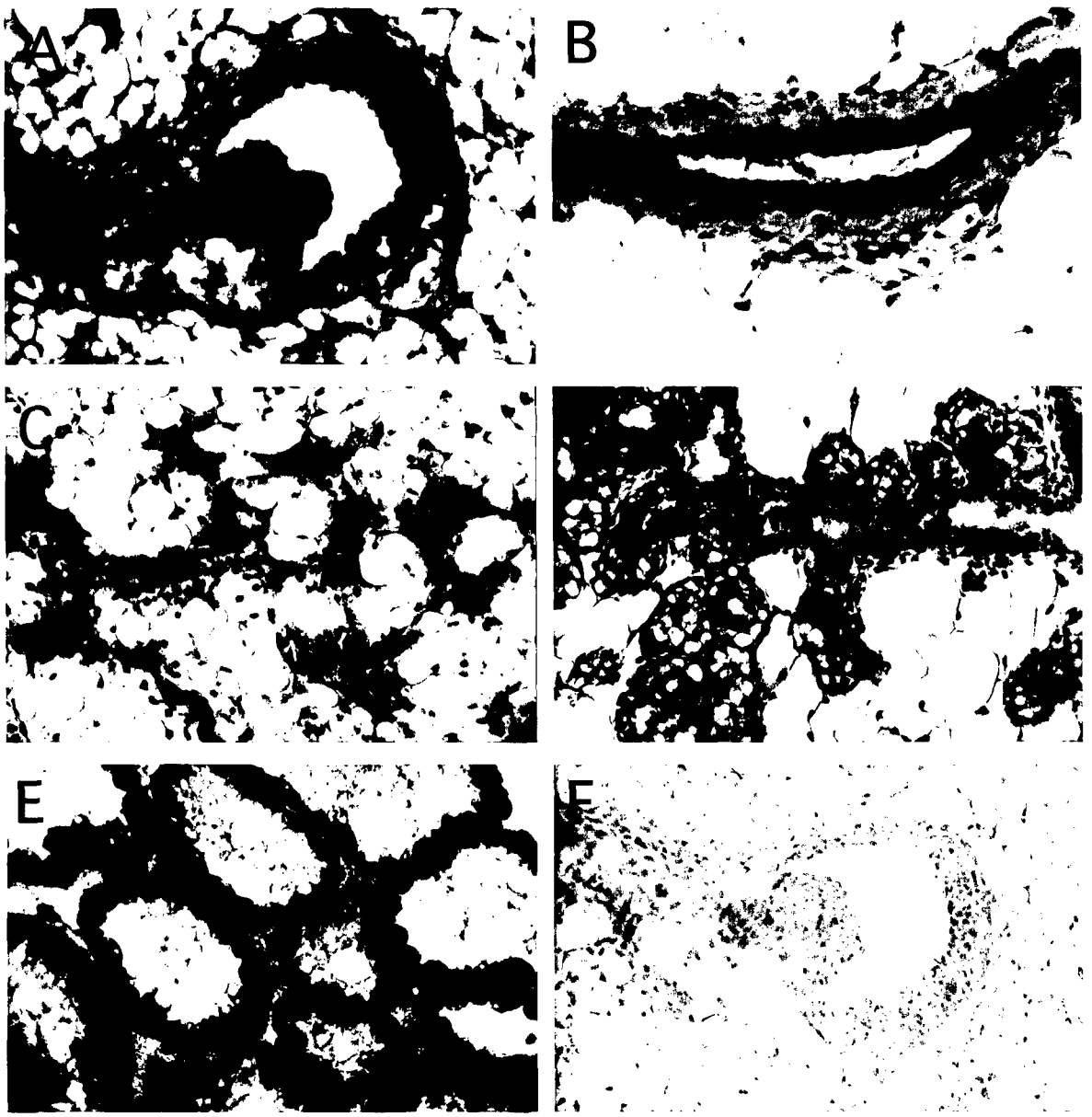

Figure 1
Page 18

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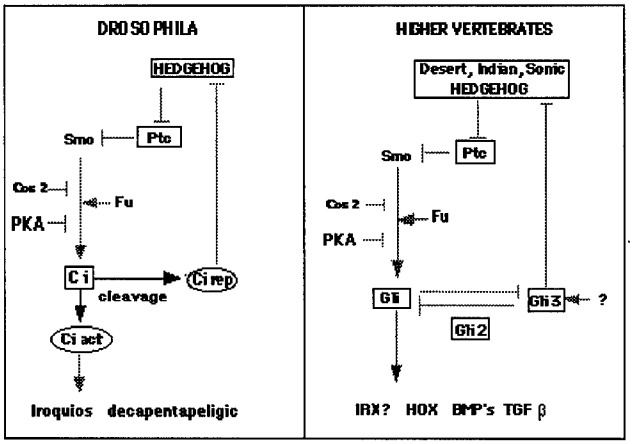

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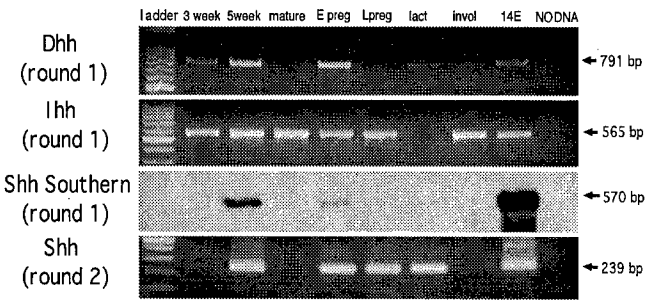

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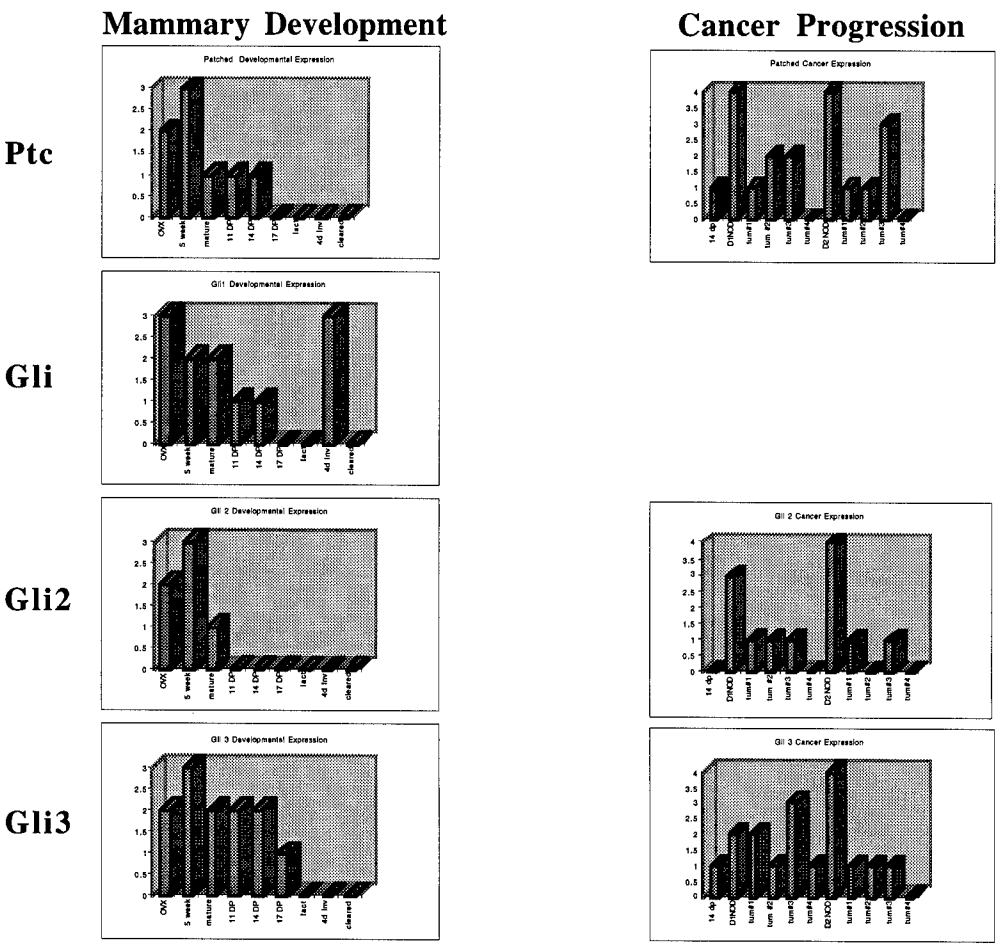

Figure 2
Page 19

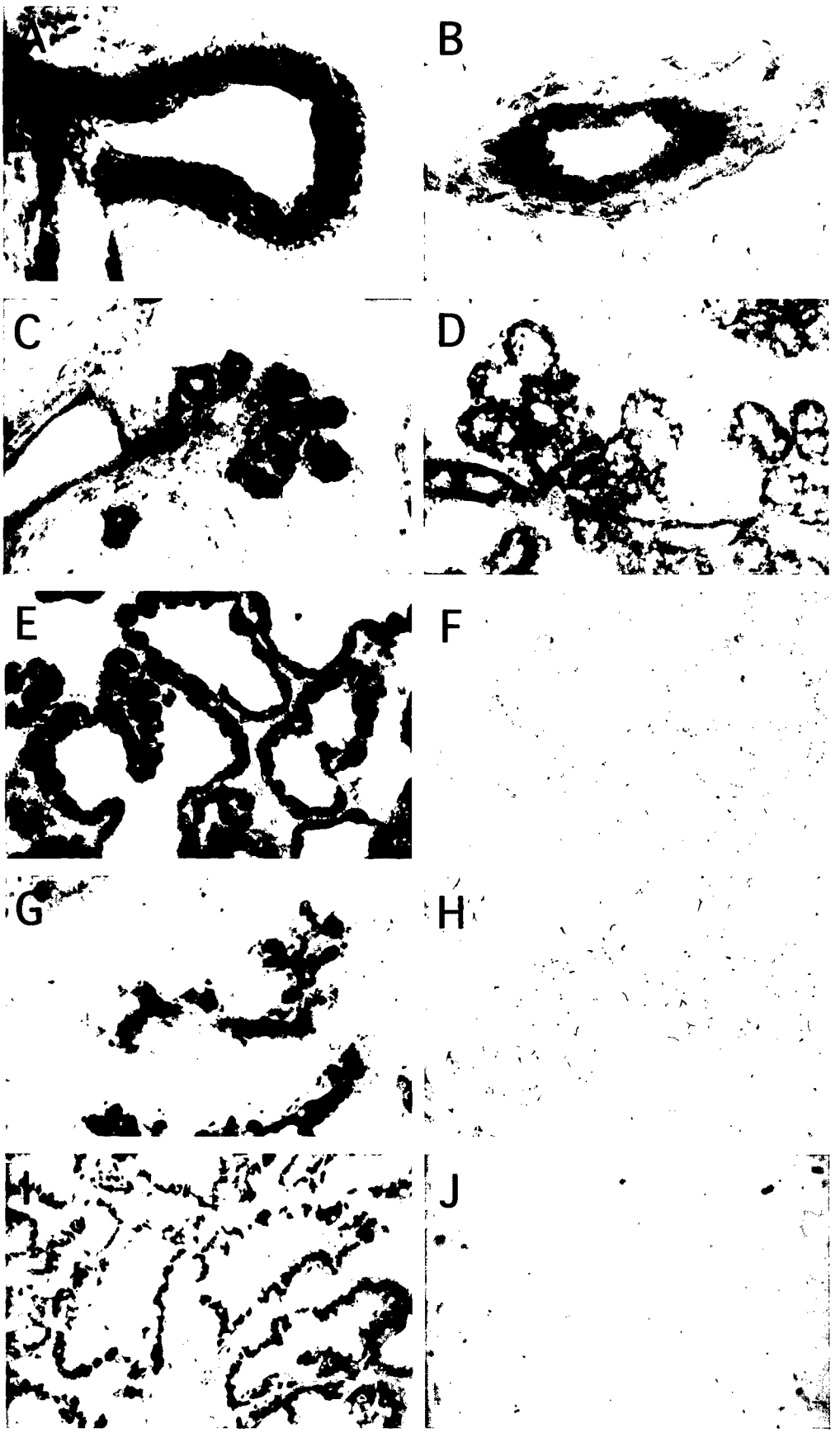

Figure 3
Page 20

FIGURE LEGENDS

Fig. 1. In situ hybridization of *Hoxd-10*. A) Terminal end bud in 5 week mouse. B) Mature mammary duct. C) Pregnancy. D) Late pregnancy. E) Lactation. F) Sense strand control

Fig. 2. RT-PCR and Northern blot analysis of hedgehog signaling pathway. A) Model of the Hedgehog signal transduction pathway for flies and mammals. B) RT-PCR expression of *Dhh*, *Ihh*, and *Shh* at different developmental stages of the mammary gland. C) Northern expression of *Ptc, Gli, Gli2,* and *Gli3* in mammary development and breast cancer progression.

Fig 3. *Ptc* in situ hybridization and immunohistochemistry. A) 5 week virgin B) 10 week mature virgin C) Early pregnant D) late pregnant E) lactating F) 2 day involuting G) 14 day involuting H) lactating. sense strand control probe. I) immunohistochemistry. lactating. J) immunohistochemistry. lactating with blocking peptide.

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